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14. ABSTRACT

NSCLC is a deadly disease that is driven by a multitude of factors. One of these factors is the epidermal growth factor receptor (EGFR). One of the most prominent molecular targeting agents to the EGFR is the antibody cetuximab. However, most patients develop resistance to this antibody. We have found in models of cetuximab resistance that the EGFR changes its location, to the nucleus, where it is not accessible to the large antibody. Our work over the last several years has discovered how to target the nEGFR, by blocking its translocation to the nucleus through Src Family Kinase blockade. In this first year we have determined 1) that nEGFR can serve as a prognostic factor in early stage NSCLC patients., 2) we have determined that we can target nEGFR in vivo and redistribute to the membrane in vivo, a critical first step for re-sensitizing to cetuximab and 3) developed a new avenue by developing a novel EGFR mutant that lacks its transcriptional potential. This will allow us to directly test the role of nEGFR in biology and cetuximab resistance.

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cetuximab resistance, nuclear EGFR, dasatinib, non-small cell lung cancer

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INTRODUCTION:

The **goals** of this proposal are to **1)** determine if targeting the nuclear EGFR (nEGFR) signaling pathway can increase the efficacy of anti-EGFR antibody based therapies in non-small cell lung cancer (NSCLC) and **2)** determine if nEGFR can serve as a prognostic factor in NSCLC.

The EGFR is a ubiquitously expressed receptor tyrosine kinase (RTK) involved in the etiology of NSCLC. With this, intense efforts have been undertaken to stop EGFR function. These efforts have been highly fruitful as four drugs, including two small tyrosine kinase inhibitors (TKIs, gefitinib and erlotinib) and two antibodies (cetuximab and panitumumab), have moved to the clinic to target EGFR in NSCLC patients. In 2004, the identification of specific genetic mutations within the EGFR kinase domain of adenocarcinomas of the lung that predict response to EGFR-TKIs represented a landmark development in the EGFR field. Unfortunately, no such mutations that predict response to cetuximab have yet been identified. Clinical trials (FLEX trial[1]) investigating cetuximab in NSCLC showed clinical benefit. However, not all patients respond to cetuximab therapy and most acquire resistance to cetuximab.

It is well established that the EGFR can rely on two distinct compartments of signaling: **1)** Classical membrane bound signaling (classical EGFR pathway)[2] and **2)** nuclear signaling (nEGFR pathway)[3]. In the nEGFR pathway, recent data suggests that **the EGFR is phosphorylated by Src family kinases (SFKs)[4,5] and AKT[6], which are necessary, early, events for trafficking EGFR from the membrane to the nucleus**. In the nucleus EGFR is able to promote the transcription of genes essential for cell proliferation and cell cycle regulation[6-12].

To explore molecular mechanisms of resistance to cetuximab in NSCLC our lab developed a series of cetuximab-resistant models using NSCLC cancer lines[13]. During investigations into potential molecular mechanisms of resistance we found that NSCLC tumor cells that acquired resistance to cetuximab had increased SFK activity [14] and increased nEGFR[5]. Further investigation revealed that SFKs regulate EGFR translocation to the nucleus[5] and the nuclear activity of EGFR contributes to resistance to cetuximab therapy[5]. However, this preliminary work has led to several questions that form the **focus** of this application: **1)** Can blocking SFK and AKT activity decrease nuclear translocation of the EGFR *in vivo*, **2)** will this lead to increased expression of EGFR on the cell membrane, **3)** will this increase sensitivity to cetuximab therapy and **4)** what is the prevalence of nEGFR in NSCLC patient biopsies and can it serve as a prognostic factor? In this proposal we ***hypothesize*** that nEGFR contributes to NSCLC resistance to cetuximab and that targeting nEGFR, by abrogating its translocation to the nucleus via SFK or AKT inhibition, followed by targeting membrane bound EGFR with cetuximab will increase therapeutic response of NSCLC tumors to cetuximab. To test this hypothesis we propose the following specific aims:

Specific Aim 1: To determine if SFK or AKT inhibition can 1) block EGFR translocation to the nucleus 2) decrease nEGFR function and 3) increase EGFR expression on the cell membrane.

Specific Aim 2: Determine if targeting nEGFR, via SFK or AKT inhibition, can increase therapeutic response of nEGFR positive, cetuximab-resistant NSCLC tumors to cetuximab.

Specific Aim 3: Determine the prevalence of nEGFR protein expression in NSCLC using IHC and AQUA/Vectra analyses and determine if it serves as a prognostic factor in NSCLC.

BODY:

In this first year of this DoD-LCRP award we have focused on two areas of the SOW; the first area being focused on Specific Aim 1 and the second on Specific Aim 3. We have also highlighted additional findings below with a thorough description.

Specific Aim 1: *To determine if SFK or AKT inhibition can **1)** block EGFR translocation to the nucleus and if this leads to decreased nEGFR function and **2)** increase EGFR expression on the cell membrane.*

In the first year we focused our energies to determine if in vivo, treatment with dasatinib could decrease nuclear levels and increase membrane levels.

We performed an experiment using HC1, HC4 and HC8 (high nEGFR) (HC4 depicted) cetuximab-resistant clone to determine if our *in vitro* findings of targeting nEGFR would translate *in vivo*. To accomplish this we inoculated the dorsal flank of athymic nude mice with cetuximab-resistant clone HC4. Mice were treated with vehicle or 70mg/kg/day of dasatinib for three days. Tumors were harvested, fixed and prepared for VECTRA imaging (*Quantitative per-cell measurement of nuclear, cytoplasmic, and membranous protein expression* for EGFR distribution (**Figure 1**))[15]. The results of this experiment indicated dasatinib treatment significantly reduced nEGFR levels, while increasing membrane/cytoplasmic EGFR levels *in vivo*. *This data provides proof-of-principle that targeting nEGFR with dasatinib leads to decreased nEGFR with subsequent increased membrane EGFR (**Figure 1**). We are now performing this work with AKT blockade to determine which form leads to better redistribution of the EGFR and decreases nEGFR in vivo.*

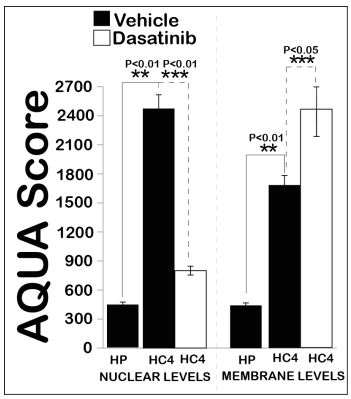


Figure 1: Dasatinib treatment of LSCC tumor xenografts leads to decreased nuclear and increased membrane EGFR (VECTRA analysis from *in vivo* tumors) **A)** Nuclear fraction **B)** Membrane fraction. P-values are indicated with ***.**

Specific Aim 3: *Determine the prevalence of nEGFR protein expression in NSCLC using IHC and AQUA analyses and determine if it serves as a prognostic factor in NSCLC.*

The focus of this aim was to use two NSCLC TMAs with various stages of NSCLC. In a first effort we focused our time on the 88 patient TMA that contained only stage I and II patients. Briefly, the findings are summarized below:

Introduction: Nuclear EGFR (nEGFR) has been identified in various human tumor tissues, including cancers of the breast, ovary, oropharynx, and esophagus, and has predicted poor patient outcomes. We sought to determine if protein expression of nEGFR is prognostic in early stage non-small cell lung cancer (NSCLC). Methods: Resected stages I and II NSCLC specimens were evaluated for nEGFR protein expression using immunohistochemistry (IHC). Cases with at least one replicate core containing ≥5% of tumor cells demonstrating strong dot-like nucleolar EGFR expression were scored as nEGFR positive.

Results: Twenty-three (26.1% of the population) of 88 resected specimens stained positively for nEGFR. Nuclear EGFR protein expression was associated with higher disease stage (45.5% of stage II vs. 14.5% of stage I; p = 0.023), histology (41.7% in squamous cell carcinoma vs. 17.1% in adenocarcinoma; p = 0.028), shorter progression-free survival (PFS) (median PFS 8.7 months [95% CI 5.1–10.7 mo] for nEGFR positive vs. 14.5 months [95% CI 9.5–17.4 mo] for nEGFR negative; hazard ratio (HR) of 1.89 [95% CI 1.15–3.10]; p = 0.011), and shorter overall survival (OS) (median OS 14.1 months [95% CI 10.3–22.7 mo] for nEGFR positive vs. 23.4 months [95% CI 20.1–29.4 mo] for nEGFR negative; HR of 1.83 [95% CI 1.12–2.99]; p = 0.014).

Conclusions: Expression of nEGFR protein was associated with higher stage and squamous cell histology, and predicted shorter PFS and OS, in this patient cohort. Nuclear EGFR serves as a useful independent prognostic variable and as a potential therapeutic target in NSCLC.

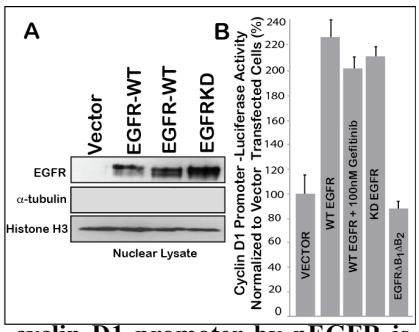
This paper was published in the journal of Lung Cancer[16] is attached. We are now currently focusing on the larger TMA with stages I-IV.

Novel findings stemming directly from this line of investigation:

During this first year efforts we took a novel approach in our laboratory to identify the key regions of the C-terminal that impart EGFRs nuclear transcriptional function[17,18]. We have provided a description of this work below.

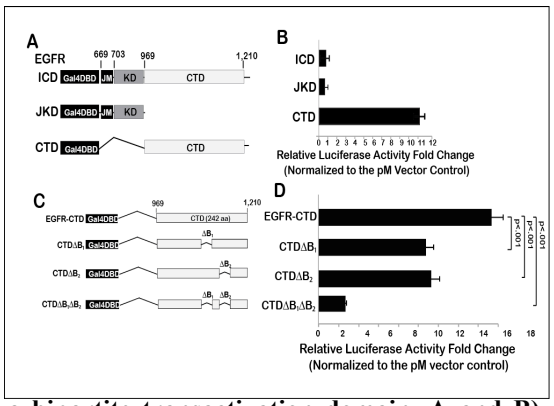
Development and characterization of nEGFR transcriptional null mutant isogenic lines; *Tools to understand nEGFR in tumor biology and cetuximab resistance.*

To date, two functions of nEGFR have been identified: **1)** the ability to phosphorylate substrates within the nucleus (kinase-dependent activity) and **2)** the ability to serve as a co-transcription factor (kinase-independent activity, **Figure 2**)[19]. To first demonstrate that nEGFR transcriptional activity is kinase-independent we expressed wildtype EGFR (EGFRWT) in CHO cells along with the Cyclin D1-Luciferase reporter gene (D1-Luc), a promoter that EGFR regulates[7], in the absence or presence of gefitinib (**Figure 2B**). The results of these experiments indicated that EGFRWT could induce D1-Luc expression and gefitinib failed to block this induction. Further, a kinase-dead EGFR (EGFRKD) control was also able to induce transcription of the D1-Luc reporter, thus strengthening the case that EGFR *transcription* is kinase-independent.



cyclin D1 promoter by nEGFR is kinase-independent. A) Nuclear EGFR mutants can enter the nucleus. **B)** EGFR transcription is kinase-independent. Luciferase assays using a minimal Cyclin D1 promoter-luciferase construct indicated that WT-EGFR blocked with gefitinib could not abrogate transcription. Further, an EGFRKD could induce transcription thus demonstrating this event is kinase-independent.

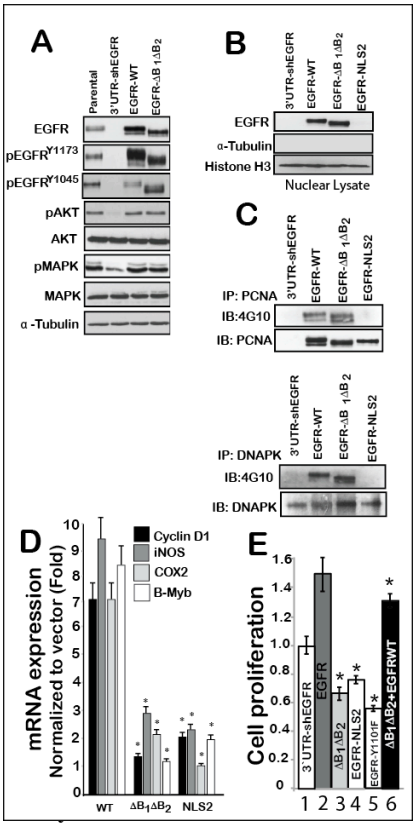
To determine if nEGFRs transcriptional activities are crucial for tumor biology and resistance to cetuximab we developed an EGFR mutant null for its transcriptional activity (**Figure 3**)[18]. To create a transcriptional EGFR mutant, we mapped the C-terminal domains necessary for transcriptional potential as we had done previously for HER3[17,18]. This approach identified two regions, which were termed B_1 and B_2, for bipartite 1 and bipartite 2, in the C-terminal of the EGFR tail that are necessary to confer transcription potential, (**Figure 3**). Deletion of these regions ($EGFR\Delta B_1\Delta B_2$) abrogated transcriptional potential of known nEGFR target genes **(Figure 3&4)**[18].



a bipartite transactivation domain. A and B) EGFR-CTD contains strong transactivation potential**.** The intracellular domain (ICD), juxtamembrane and kinase domain (JKD) and C-terminal domain (CTD) of EGFR were fused to the Gal4 DNA binding domain (Gal4DBD). CHOK1 cells were transfected with EGFR-ICD, EGFR-JKD or EGFR-CTD constructs and luciferase assays performed per instruction. **C and D)** EGFR contains a strong bipartite C-terminal transactivation domain. After mapping the CTD we identified two critical sites, B_1 and B_2 for transcription. CHOK1 cells were transfected with EGFR-CTD, $CTD\Delta B_1$, $CTD\Delta B_2$ or $CTD\Delta B_1\Delta B_2$ constructs and Cyclin-D1 luciferase reporter and luciferase assays performed per instruction.

This nEGFR transcriptional mutant, and several controls, were used to create an isogenic cell system in the LSCC NCI-H226 cell parental cell line (**Figure 4**). This line was chosen for several reasons: **1)** it is the SCC line where we developed and investigated cetuximab resistance in LSCC, **2)** H226 has been used by several groups to investigate metastatic spread using mouse model systems[20] and **3)** this line gives up to 75% metastatic lung lesions when used in flank xenograft systems[21]. To create an isogenic model system we used the H226 parental line and stably knocked down EGFR expression using a 3`-UTR shEGFR vector (A kind gift from Jeff Settleman[22]). This line was then used to *add back* nEGFR mutant variants to create an isogenic system that includes: **1)** Parental H226, **2)** 3`UTR shEGFR, **3)** EGFRWT, **4)** $EGFR\Delta B_1\Delta B_2$ and **5)** EGFR-NLS2 (nuclear deficient EGFR[23]) (**Figure 4A**). **Controls**: We will make use a known fully activated, nuclear deficient EGFR mutant, EGFR-NSL2[23,24], where the EGFR NLS was mutated from a "RRRHIVRKRTLRR" to a RRRHIVAAATLRR rendering it deficient for nuclear entry. In addition, in order to study predominantly nuclear signaling all constructs were fused to the triplicate SV40-NLS sequence to increase the nuclear levels of EGFR, a technique previously used in our lab[5]. Characterization of this model membrane signaling as indicated by activation of MAPK and AKT (**Figure 4A**) Further, EGFRWT and $EGFR\Delta B_1\Delta B_2$ are nuclear localized, whereas EGFRNLS2 was deficient (**Figure 4B**). In addition, both the EGFRWT and $EGFR\Delta B_1\Delta B_2$ phosphorylate known nuclear substrates of the EGFR PCNA[25] and DNAPK[26], respectively, whereas the EGFRNLS2 was unable to enter the nucleus and phosphorylate known EGFR substrates (DNAPK and PCNA) (**Figure 4C**). Next we tested if the $EGFR\Delta B_1\Delta B_2$ was transcriptionally null for known EGFR target genes including Cyclin D1, iNOS, COX2 and B-Myb. The results of this experiment, using

qPCR, indicated that transfection of EGFRWT was able to increase transcription of nEGFR target genes whereas EGFR$\Delta B_1\Delta B_2$ and EGFRNLS were not (**Figure 4D**). Finally, it was determined that the EGFR$\Delta B_1\Delta B_2$ impaired proliferative potential (**Figure 4E**). *Collectively, this isogenic system will enable us to better define the role of nEGFR in NSCLC.*



transcription in tumor biology and cetuximab resistance. A) EGFRWT and EGFRΔB1ΔB2 are active and activate the MAPK and AKT pathways. **B)** EGFRWT and EGFRΔB1ΔB2 are nuclear localized, whereas, EGFRNLS2 is nuclear deficient. **C)** EGFR$\Delta B_1\Delta B_2$ but not EGFRNLS2 can phosphorylate known nEGFR targets, PCNA and DNAPK. **D)** EGFR$\Delta B_1\Delta B_2$ is deficient in activating expression of known EGFR target genes relative to EGFRWT. **E)** EGFR$\Delta B_1\Delta B_2$ exhibits slower proliferation relative to EGFRWT controls (2), or two nuclear deficient EGFR mutants EGFR-NLS2 (4) and EGFRY1101F (5). Proliferation of the EGFR$\Delta B_1\Delta B_2$ stable line can be ***rescued*** by adding back EGFRWT (6).

KEY RESEARCH ACCOMPLISHMENTS:

- We have demonstrated that tumors that harbor high levels of nEGFR can targeted by dasatinib, which leads to the increased levels on the membrane. (Aim 1 SOW)
- We have determined that nEGFR is a prognostic factor in stage I and II NSCLC. (Aim 3 SOW)
- We have developed a novel EGFR mutant that is kinase functional, but transcriptionally null. We did this through a series of mapping experiments. This will allow us to directly test the role of nEGFR transcriptional function in biology and cetuximab resistance.

REPORTABLE OUTCOMES:

- manuscripts, abstracts, presentations;
  - Traynor, A.M.*, et al.* Nuclear EGFR protein expression predicts poor survival in early stage non-small cell lung cancer. *Lung cancer* **81**, 138-141 (2013).
  - Brand, T.M., Iida, M., Luthar, N. & Wheeler, D.L. Mapping the transcriptional activation domains of the HER family of receptor tyrosine kinases. in *American Association of Cancer Research* (Washington, DC, 2013).
- licenses applied for and/or issued N/A
- degrees obtained that are supported by this award; N/A/
- development of cell lines, tissue or serum repositories; N/A
- infomatics such as databases and animal models, etc.; N/A
- funding applied for based on work supported by this award;

  With the funding from this DoD award we developed a novel EGFR mutant that is null for its transcriptional functions. This has allowed us to ask new and novel questions centered on the role of nEGFR transcription and its role in NSCLC tumor biology (angiogenesis, metastasis, transformation, etc) and cetuximab resistance. This approach and questions was recently submitted to the DoD-LCRP entitled "*Bifurcation of nuclear EGFR function to elucidate its role in tumor biology and cetuximab resistance of the lung*" and was invited for a full application. We are extremely excited about this opportunity to continue our efforts to understand the role of nEGFR in lung cancer.
- employment or research opportunities applied for and/or received based on experience/training supported by this award N/A

CONCLUSION:

NSCLC is a deadly disease that is driven by a multitude of factors. One of these factors is the epidermal growth factor receptor (EGFR). One of the most prominent molecular targeting agents to the EGFR is the antibody cetuximab. However, most patients develop resistance to this antibody. We have found in models of cetuximab

resistance that the EGFR changes its location, to the nucleus, where it is not accessible to the large antibody. Our work over the last several years has discovered how to target the nEGFR, by blocking its translocation to the nucleus through Src Family Kinase blockade.

In this first year we have determined that nEGFR can serve as a prognostic factor in early stage NSCLC patients. We are building on this finding to see if nEGFR can serve as a prognostic factor for late stage patients, a goal of Aim 3. Secondly we have determined that we can target nEGFR in vivo and redistribute to the membrane in vivo, a critical first step for re-sensitizing to cetuximab. Finally, we have developed a new avenue by developing a novel EGFR mutant that lacks its transcriptional potential. This will allow us to directly test the role of nEGFR in biology and cetuximab resistance.

We have made no medical products during the first year of this experimentation.

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APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, study questionnaires, and surveys, etc.

Traynor, A.M*., et al.* Nuclear EGFR protein expression predicts poor survival in early stage non-small cell lung cancer. *Lung cancer* **81**, 138-141 (2013).

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Early report

Nuclear EGFR protein expression predicts poor survival in early stage non-small cell lung cancer

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ABSTRACT

Introduction: Nuclear EGFR (nEGFR) has been identified in various human tumor tissues, including cancers of the breast, ovary, oropharynx, and esophagus, and has predicted poor patient outcomes. We sought to determine if protein expression of nEGFR is prognostic in early stage non-small cell lung cancer (NSCLC).
Methods: Resected stages I and II NSCLC specimens were evaluated for nEGFR protein expression using immunohistochemistry (IHC). Cases with at least one replicate core containing ≥5% of tumor cells demonstrating strong dot-like nucleolar EGFR expression were scored as nEGFR positive.
Results: Twenty-three (26.1% of the population) of 88 resected specimens stained positively for nEGFR. Nuclear EGFR protein expression was associated with higher disease stage (45.5% of stage II vs. 14.5% of stage I; $p = 0.023$), histology (41.7% in squamous cell carcinoma vs. 17.1% in adenocarcinoma; $p = 0.028$), shorter progression-free survival (PFS) (median PFS 8.7 months [95% CI 5.1–10.7 mo] for nEGFR positive vs. 14.5 months [95% CI 9.5–17.4 mo] for nEGFR negative; hazard ratio (HR) of 1.89 [95% CI 1.15–3.10]; $p = 0.011$), and shorter overall survival (OS) (median OS 14.1 months [95% CI 10.3–22.7 mo] for nEGFR positive vs. 23.4 months [95% CI 20.1–29.4 mo] for nEGFR negative; HR of 1.83 [95% CI 1.12–2.99]; $p = 0.014$).
Conclusions: Expression of nEGFR protein was associated with higher stage and squamous cell histology, and predicted shorter PFS and OS, in this patient cohort. Nuclear EGFR serves as a useful independent prognostic variable and as a potential therapeutic target in NSCLC.

1. Introduction

Non-small cell lung cancer is a heterogeneous malignancy, comprised of multiple histologic subtypes. Predicting the course of disease based upon staging is suboptimal. The identification of biological markers of aggressive clinical behavior is needed in an effort to individualize treatment and develop novel therapeutic targets.

Protein expression of membrane bound EGFR was neither prognostic nor predictive of efficacy with the use of erlotinib, gefitinib, or cetuximab in NSCLC [1,2]. However, emerging preclinical and clinical evidence supports the role of nEGFR in enhancing tumor cell growth, survival, and resistance to systemic and radiation therapies [3–10]. Herein, we report identification of nEGFR protein expression as an independent prognostic variable in early stage NSCLC.

2. Materials and methods

2.1. Patients and specimen collection

For this retrospective analysis of patients who underwent curative intent resections, de-identified tumor specimens from 88 deceased patients with stages I and II NSCLC were collected from the University of Wisconsin Hospitals and Clinics (UWHC; Madison, WI) and from the Gundersen Lutheran Medical Center (GLMC;

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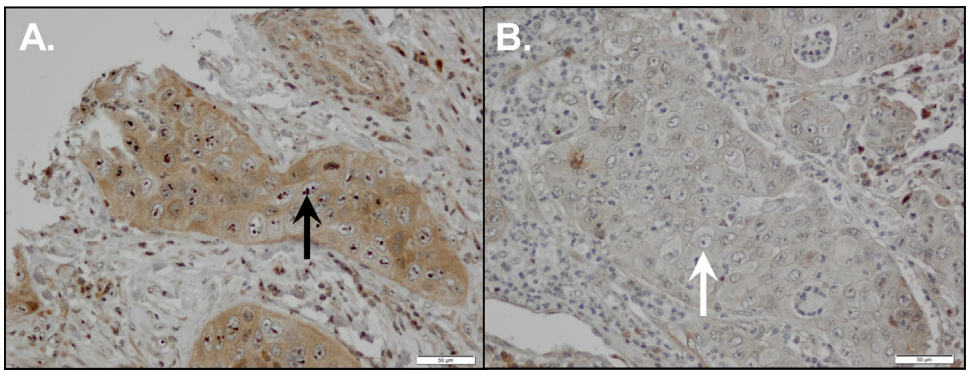

Fig. 1. Nuclear EGFR (nEGFR) is detected in early stage NSCLC specimens. We analyzed 88 primary NSCLC tumors for nEGFR protein expression using immunohistochemistry. (A) Representative case demonstrating nEGFR expression. All positive cases had a similar distinctive pattern of strong nucleolar staining (black arrow). (B) Representative case demonstrating a lack of nEGFR protein expression. Despite the presence of prominent nucleoli, no nEGFR protein is detected (white arrow).

LaCrosse, WI). Patients did not receive either pre- or post-operative anti-cancer therapy. We also collected: age, sex, histology, smoking history, pathologic stage (AJCC Staging 6th edition), type of resection, date of relapse, and date of death. Approval for this research was obtained from the IRBs of UW-Madison and the GLMC.

2.2. Tissue microarray construction and protein expression analyses

Tumor tissue quality and pathology were confirmed by the study pathologist (DTY). Tissues were harvested within 30 min of resection, fixed with 10% neutral buffered formalin and embedded in paraffin. Areas of tumor and adjacent benign tissue were marked on a representative H & E stained section. Duplicate 0.6 mm cores from the corresponding paraffin block were punched out and assembled with a Manual Tissue Arrayer (Beecher Instruments, Sun Prairie, WI).

For nEGFR protein expression analyses, tissue sections were deparaffinized and antigen retrieval was performed in citrate buffer (pH 6.0) with 0.05% Tween-20. Samples were incubated with EGFR polyclonal antibody (sc-03, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4C. Samples were washed and incubated in secondary antibody for 1 hour followed by incubation with Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). 3,3-Diaminobenzidine staining was used as the color-developing reagent. Slides were counterstained with Mayer hematoxylin, dehydrated through a graded series of ethanol washes to xylene, and coverslipped with Permount (Fisher, Springfield, NJ).

We initially hypothesized that assessment of nEGFR protein would require the quantitative and subcellular localization capacity of automated quantitative analysis (AQUA). When we observed that the nuclear staining of EGFR protein revealed a distinct, robust nucleolar pattern (Fig. 1A) that clearly contrasted with negative cases (Fig. 1B) using routine IHC staining, we switched to the IHC methodology due to its easier translation to clinical practice. The nEGFR staining pattern was scored by the study pathologist at 5% increments by visual estimation at 20× magnification. Accordingly, cases with at least one replicate core containing at least 5% of tumor cells demonstrating strong dot-like nucleolar EGFR IHC protein expression were scored as nEGFR positive.

2.3. Statistical analyses

Our endpoints were protein expression of nEGFR and PFS and OS. Originally this study had an approximate power of 0.902, 0.747 and 0.477 to detect a hazard ratio of 2, 1.75 and 1.5, respectively, using a two-sided log-rank test at a significance level 0.05, given the sample size of 88 when the AQUA score was dichotomized using its median. The prognostic impact of nEGFR was assessed using the log-rank test and Cox proportional hazards regression models for PFS and OS. Kaplan–Meier method was used to summarize PFS and OS for patients per nEGFR IHC. Association between nEGFR protein expression and sex, histology, smoking history and pathologic stage was assessed using Fisher's exact test.

3. Results

3.1. Patient characteristics

Table 1 summarizes the characteristics of the 88 patient samples studied. None of the patients received either pre- or post-operative anti-cancer therapy. The median PFS and OS for our population

Table 1
Patient characteristics.

	N
Number of patients	88
Median age (range)	73 (43–96 yrs)
Sex	
Male	55 (62.5%)
Female	33 (37.5%)
Histology	
Adenocarcinoma	41 (46.6%)
Squamous cell	36 (40.9%)
Bronchioloalveolar	4 (4.5%)
Large cell	3 (3.4%)
Non-small cell, NOS	2 (2.3%)
Adenosquamous carcinoma	2 (2.3%)
Smoking history	
Current or former	84 (95.5%)
Type of surgery	
Lobectomy	80 (90.9%)
Pneumonectomy	7 (8%)
Bilobectomy	1 (1.1%)
Disease stage	
IA	23 (26.1%)
IB	32 (36.4)
IIA	9 (10.2%)
IIB	24 (27.3%)
T stage	
T1	31 (35.2%)
T2	52 (59.1%)
T3	5 (5.7%)
N stage	
N0	60 (68.2%)
N1	28 (31.8%)
Nuclear EGFR protein expression	
Positive	23 (26.1%)

Table 2
Distribution of nuclear EGFR protein staining per IHC across all tumor specimens.

Patient number	Percent of cells with positive nuclear EGFR protein staining per IHC			
	Cores (all specimens run in duplicate when tissue available)			
	Tumor 1	Tumor 2	Adjacent normal lung 1	Adjacent normal lung 2
1	50	NC	0	0
2	80	60	0	0
3	80	20	0	0
4	50	75	0	0
5	95	50	0	0
6	25	25	0	0
7	0	5	0	0
8	60	20	NC	NC
9	10	5	0	0
10	60	50	NC	NC
11	20	30	0	0
12	30	80	0	0
13	5	10	0	0
14	80	90	0	0
15	15	5	0	0
16	30	100	0	0
17	20	NC	0	0
18	60	70	0	0
19	40	70	0	0
20	90	90	0	0
21	30	NC	NC	NC
22	40	60	0	0
23	30	5	0	0
Specimens from remaining 65 patients	0	0	0	0

NC, no core available.

were 11.3 months (95% CI 9.1–16.2 mo) and 22.0 months (95% CI 15.9–24.7 mo), respectively, shorter than expected. Fifty-nine patients experienced disease relapse. Since only four patients were non-smokers, and seven underwent a pneumonectomy, these two clinical characteristics were dropped from further analyses.

Twenty-three (26.1% of the population) of 88 patients had specimens that stained positively for nEGFR (Fig. 1A). When nEGFR expression was seen, greater than 40% of tumor cells were positive in most cases. Nuclear EGFR was seen in between 1% and 4% of tumor cells very rarely (4/165 tumor cores). Control cores comprised of EGFR positive ductal carcinoma of the breast and matched adjacent normal lung from each tumor were represented on the TMA as external and internal controls, respectively. Cytoplasmic and membrane EGFR staining were confirmed in the breast control, and no nEGFR expression was observed in any of the adjacent normal lung tissue. Table 2 depicts the distribution of nEGFR positivity per IHC staining across our tumor samples.

3.2. Nuclear EGFR protein expression and survival

According to the log-rank test, nEGFR protein positivity was associated with shorter PFS (median PFS 8.7 months [95% CI 5.1–10.7 mo] for nEGFR positive vs. 14.5 months [95% CI 9.5–17.4 mo] for nEGFR negative; HR = 1.89 [95% CI 1.15–3.10]; $p = 0.011$), and shorter OS (median OS 14.1 months [95% CI 10.3–22.7 mo] for nEGFR positive vs. 23.4 months [95% CI 20.1–29.4 mo] for nEGFR negative; HR = 1.83 [95% CI 1.12–2.99]; $p = 0.014$).

3.3. Nuclear EGFR protein expression and prognosis

According to Fisher's exact test, nEGFR protein positivity was associated with squamous cell histology, compared to adenocarcinoma (nEGFR positive in 41.7% of patients' samples with squamous cell vs. 17.1% in adenocarcinoma specimens, $p = 0.028$), and with higher disease stage (nEGFR positive in 45.5% of stage II vs. 14.5% of stage I, $p = 0.023$). Nuclear EGFR protein expression was not associated with patient's sex, or T or N status.

According to Cox proportional hazard models, of the baseline clinical characteristics (sex, disease stage, histology, T, N, and age), only age was at least marginally associated with PFS ($p = 0.073$), but was not associated with OS. Also nEGFR protein positivity in patients' specimens was associated with shorter PFS, after controlling for age, with an HR of 1.68 (95% CI 1.01–2.81, $p = 0.046$), and with shorter OS with an HR of 1.83 (95% CI 1.12–2.99, $p = 0.016$).

4. Discussion

Nuclear EGFR was first observed in hepatocytes during liver regeneration. Translocation from the cell membrane to the nucleus has been reported with numerous receptor tyrosine kinases (RTKs), including all HER family receptors, MET, and VEGFR2 [3,4]. Protein expression of nEGFR has correlated with shortened survival in cancers of the breast, ovary, and oropharyngeal and esophageal squamous cells. Approximately 25–50% of the tumor cells expressed nEGFR [5–8].

Nuclear translocation of full length EGFR can be initiated by ligand binding, irradiation, cetuximab, and cisplatin [4,9,10]. Early events for movement of EGFR from the plasma membrane to the nucleus include phosphorylation of the dimerized receptor by SRC family kinases and AKT [10,11]. These stimuli induce internalization to endocytic vesicles. EGFR then undergoes retrograde translocation through the Golgi apparatus to the endoplasmic reticulum, whereupon it moves from the outer nuclear membrane to the inner nuclear membrane via interaction between importin β and the nuclear pore complex. In the inner nuclear membrane, EGFR can interact with Sec61 for removal from the membrane and release into the nucleus [4,12].

Within the nucleus three functions have been identified for the EGFR. First, EGFR associates with STAT3, STAT5 and E2F1 to act as a transcriptional co-activator, independent of its kinase activity, to increase the expression of target genes that worsen

the malignant phenotype (cyclin D1, iNOS, B-myb, c-Myc, Aurora kinase A, Breast Cancer Resistance Protein, and COX-2) [3,4,13]. Second, nEGFR phosphorylates proliferating cell nuclear antigen, promoting DNA replication [14]. Third, it activates DNA-dependent protein kinase within the nucleus, stimulating DNA repair following exposure to irradiation and cisplatin [15].

This study demonstrates that a distinct nucleolar pattern of EGFR protein was associated with significantly shorter PFS and OS, higher stage and squamous histology in patients with early stage NSCLC. These correlations were not confounded by exposure to additional anti-cancer therapies. A limitation of our study is our shorter than expected overall survival; this is most certainly related to the fact that all samples were selected from patients who had expired by the time of our analyses. Within our patient cohort, however, nEGFR protein expression was detected in just over a quarter of our samples and was statistically associated with higher stage and squamous histology. These results are consistent with findings from other disease sites [5–8].

Our group, and others, have shown in experimental models that nEGFR contributes to treatment resistance with cetuximab, gefitinib, erlotinib, and irradiation [10,11,15]. For example, we demonstrated that NSCLC cells that developed acquired resistance to cetuximab expressed increased levels of nEGFR, and that forced expression of nEGFR rendered cetuximab-sensitive cells resistant to cetuximab, both in vitro and in vivo [3,10]. Similarly, Liccardi et al. showed that cells expressing EGFR with mutations that impair nuclear transport demonstrated reduced repair of DNA strand breaks following ionizing radiation and reduced repair of inter-strand cross-links following exposure to cisplatin, as compared to cells capable of directing EGFR to the nucleus [15]. Conversely, sensitivity in cetuximab-resistant NSCLC cells was re-established after blocking nuclear translocation of EGFR by co-exposing cells to either dasatinib, a SRC family kinase inhibitor, or MK2206, an AKT inhibitor [10,11].

Investigating the functions of nuclear RTKs in untreated cancer cells also serves as a focus of research [16]. Using sequential immunoprecipitation and immunoelectron microscopy assays, Li and colleagues demonstrated that ErbB2 co-localizes with β-actin and RNA polymerase-I (RNA Pol I) to the nucleoli in multiple breast cancer cell lines. Activation of this complex enhanced binding of RNA Pol I to rDNA, expediting rRNA synthesis and protein translation. These authors proposed that localization of ErbB2 to the nucleus and nucleoli contributed to tumorigenesis by increasing rRNA synthesis and protein translation. Nuclear EGFR has been identified in multiple tumor types in patients who did not undergo prior EGFR inhibiting therapy [5–8], as was the case with our population. Biological mechanisms that signal localization of EGFR to the nucleolus in untreated patients, as well as the potential role of such localization in tumor development, are under study in our laboratory.

5. Conclusion

We have identified nEGFR as a predictor of shortened survival in patients with early stage NSCLC. Preclinical data highlights the kinase dependent and independent processes by which nEGFR stimulates tumor cell growth, progression, and survival [3,4,10,11]. This raises the question of whether or not nEGFR represents not only a useful prognostic factor in NSCLC, but also a potential therapeutic target. The biological functions of nEGFR, and strategies to improve the efficacy of cetuximab, cisplatin and radiation by disrupting nuclear translocation of EGFR, remain the subjects of our translational research efforts.

Conflict of interest

No author of this article had any financial or personal relationships with other people or organizations that could inappropriately influence or bias this article.

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